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Chemical analysis of a new fucoglucan isolated from an edible mushroom, *Termitomyces robustus*

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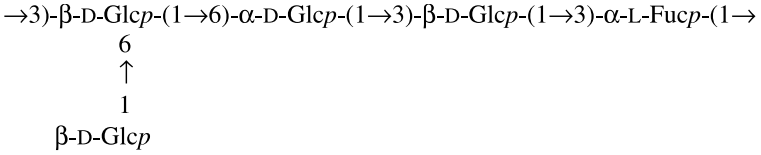
Abstract—A water-soluble fucoglucan, isolated from alkali-treated edible mushroom, *Termitomyces robustus*, consists of L-fucose and D-glucose in a molar proportion of 1:4. Structural investigation of the polysaccharide was carried out by using total hydrolysis, methylation analysis, and periodate oxidation followed by GLC–MS, and the final structure was determined using NMR experiments (^{1}H, ^{13}C, DQF-COSY, TOCSY, NOESY, ROESY, HMQC, and HMBC). On the basis of the above experiments it is concluded that the following repeating unit is present in the polysaccharide:



Keywords: *Termitomyces robustus*; Polysaccharide; Mushroom; Structure; NMR spectroscopy

1. Introduction

In recent years mushroom polysaccharides have drawn the attention of chemists and immunobiologists on account of their immunomodulatory and antitumor[1–3] properties. Polysaccharides isolated from the edible mushrooms, *Termitomyces eurhizus*,[4] *Termitomyces striatus*,[5] and *Termitomyces microcarpus*,[6] have been reported by our group. It is reported that several β-(1→3)-glucans,[7–10] β-(1→3) (1→6)-glucans,[11] β-(1→6)-glucan,[12,13] both α-(1→4),β-(1→6)-glucan[14] and α-(1→4)(1→6)-glucan,[14] and α-(1→3),β-(1→6)-glucan[11] are widely used as antitumor and immunostimulating agents. Some linear glucans,[4,6,15–18] branched glucans,[17–19] and heteroglycans[20–22] have been reported by our group in this journal. Other heteropolysaccharides such as fucogalactan,[23] fucomannogalactan,[24] and mannogalactofucan[25] show similar properties. *Termitomyces robustus* and *Termitomyces clypeatus* were found to contain[26] 31% protein, 32% carbohydrate, and 10–14% ascorbic acid. The nutrient content of *T. robustus*[27,28] has been analyzed, but no research relating to the polysaccharide of *T. robustus* has been reported. We are reporting herein for the first time the chemical analysis of a fucoglucan isolated from an edible mushroom, *T. robustus*.

2. Results and discussion

The fresh edible fruit bodies of mushroom, *T. robustus* (1.5 kg), were washed several times with water and then with distilled water, followed by extraction with hot

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water for 6 h, and then the material was kept overnight at 4 °C and centrifuged. The supernatant was precipitated in alcohol, and the residue was dissolved in 4% NaOH, dialyzed, and freeze-dried to yield 1.2 g of material. The crude water-soluble polysaccharide (30 mg) was passed through a Sepharose 6B column in aqueous medium, and a single fraction was obtained (20 mg). Separation was carried out in five lots, yielding a total of 104 mg. The molecular weight of this polysaccharide fraction was estimated as $\sim 2 \times 10^6$ Da, as determined from a calibration curve using standard dextran.[29] A detailed structural study was carried out with this polysaccharide fraction, and the outcome is reported in this paper. The specific rotation of this fraction was found to be $[\alpha]_D^{25}$ −14.96 (*c* 0.08, water, 28 °C). The total carbohydrate was estimated to be 96.3% by the phenol–sulfuric acid[30] method. Protein was determined as 3% by Lowry's method.[31] On hydrolysis with 2 M trifluoroacetic acid, followed by alditol acetate preparation and analysis through a GLC using columns A (3% ECNSS-M) and B (1% OV-225) indicated the presence of glucose and fucose. The absolute configuration of the sugars was determined by the method of Grewig et al.,[32] and it was found that glucose and fucose have the D and L configurations, respectively. The polysaccharide was methylated twice by the procedure of Ciucanu and Kerek,[33] and then by the Purdie[34] method, followed by hydrolysis and alditol acetate preparation. The mode of the linkages of the polysaccharide was determined through GLC using columns A and B. The alditol acetates were then further analyzed by GLC–MS using an HP-5 fused silica capillary column. The polysaccharide showed the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol; 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol; 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol; 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol; and 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-L-fucitol in a molar ratio of 1:1:1:1:1 (Table 1). From the above experimental results, the linkage of the constituent sugars was identified as the nonreducing-end D-glucopyranosyl, (1→3)-linked D-glucopyranosyl, (1→6)-linked D-glucopyranosyl, (1→3,6)-linked D-glucopyranosyl, and (1→3)-linked D-fucopyranosyl moieties. A further linkage confirmation was carried out by periodate oxidation. The GLC analysis of the alditol acetate derived from the periodate oxidation and reduction, followed by the methylation of the fraction, showed the presence of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol; 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol; and 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-L-fucitol in a molar ratio of 1:1:1. This result indicates that the nonreducing D-glucopyranosyl and the (1→6)-linked D-glucopyranosyl moieties are consumed during periodate oxidation. Thus the periodate oxidation experiment confirms the linkage of the sugar moieties present in the polysaccharide.

The FTIR spectrum of the polysaccharide showed bands at 850 cm^{-1} and 900 cm^{-1} corresponding to the presence of both α and β configurations, respectively. In addition to these characteristic bands, the spectrum showed bands also at 1039, 1076, 1159, and 1120 cm^{-1} owing to the presence of (1→3)-di-O-substituted glucose residues.[18]

The 500-MHz ^1H NMR (Fig. 1), 125-MHz ^{13}C (Fig. 2) and proton-coupled ^{13}C NMR experiments were carried out at 27 °C. The 500-MHz ^1H NMR spectrum of the polysaccharide showed the presence of five anomeric proton signals at δ 5.22, 5.00, 4.64, 4.53, and 4.52 in a ratio of nearly 1:1:1:1:1. All the proton chemical shifts (Table 2) and carbon chemical shifts (Table 2) were assigned using 2D-DQF-COSY, TOCSY, and HMQC experiments. The five glycosyl moieties were designated as **A**, **B**, **C**, **D**, and **E** according to their decreasing anomeric chemical shifts.

Residue **A** was assigned as an L-fucopyranosyl unit. This is strongly supported by the appearance of a proton signal at δ 1.24 and a carbon signal at δ 16.2 for a CH_3 group. The appearance of the anomeric proton signal for residue **A** at δ 5.22 and the coupling constant value of $^3J_{1,2}$ (3.75 Hz) clearly indicate that L-fucose is α-linked. This anomeric configuration was further confirmed by ^1H–^{13}C coupling constant $^1J_{H\text{-}1,C\text{-}1}$ 171 Hz. The anomeric carbon signal of residue **A** at δ 92.50 was confirmed by the presence of a cross-peak **A**

Table 1. GLC and GLC–MS data for the alditol acetates derived from the methylated PS and IO_4^-PS isolated from *T. robustus*

Compound	Methylated sugar	T^a	T^b	Characteristic fragments[c] (*m*/*z*)	Molar ratio	Mode of linkage
PS	2,3,4,6-Me_4-Glc*p*	1.00	1.00	**101**, 117, 129, 161	1	Glc*p*-(1→
	2,3,4-Me_3-Glc*p*	2.49	2.22	99, **101**, 117, 129	1	→6)-Glc*p*-(1→
	2,4,6-Me_3-Glc*p*	1.95	1.82	**43**, 101, 117, 129, 161	1	→3)-Glc*p*-(1→
	2,4-Me_2-Glc*p*	5.10	4.21	**43**, 87, 117, 129, 189	1	→3,6)-Glc*p*-(1→
	2,4-Me_2-Fuc*p*	1.12	1.02	89, 101, **117**, 131	1	→3)-Fuc*p*-(1→
IO_4^-PS	2,4,6-Me_3-Glc*p*	1.95	1.82	**43**, 101, 117, 129, 161	1	→3)-Glc*p*-(1→
	2,4-Me_2-Glc*p*	5.10	4.21	**43**, 87, 117, 129, 189	1	→3,6)-Glc*p*-(1→
	2,4-Me_2-Fuc*p*	1.12	1.02	89, 101, **117**, 131	1	→3)-Fuc*p*-(1→

The bold values signifie the more intense peak of mass value.

[a] Retention time with respect to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on a 3% ECNSSM column on a Gaschrom-Q at 170 °C.

[b] Retention time with respect to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on a 1% OV-225 column on a Gaschrom-Q at 170 °C.

[c] Equipped with a HP-5-fused-silica capillary column using a temperature program from 150 °C (2 min) to 200 °C (5 min) at 2 °C min^{-1}.

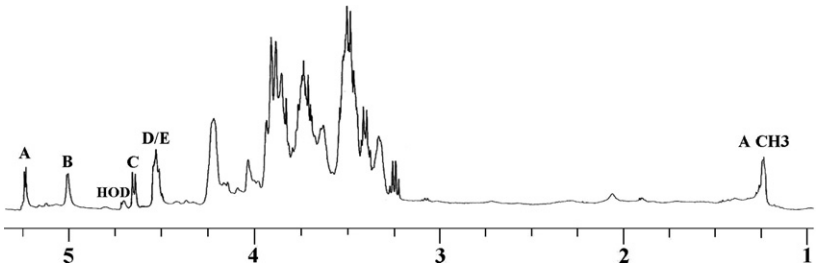


Figure 1. ^{1}H NMR spectrum (500 MHz, D_2O, 27 °C) of the polysaccharide isolated from *T. robustus*.

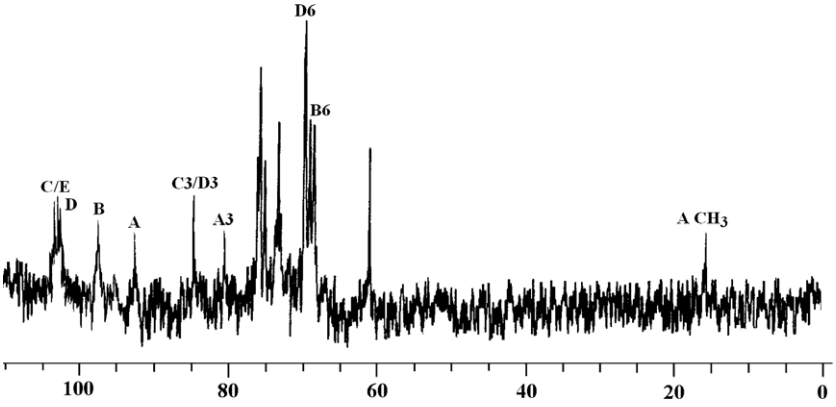


Figure 2. ^{13}C NMR spectrum (125 MHz, D_2O, 27 °C) of the polysaccharide isolated from *T. robustus*.

Table 2. ^{1}H and ^{13}C NMR chemical shifts of polysaccharide recorded in D_2O at 27 °C for *T. robustus*[a,b]

Sugar residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6a/C-6	H-6b
→3)-α-L-Fuc*p*-(1→	5.22	3.53	3.32	3.85	4.21	1.24	
A	92.50	68.25	80.50	69.28	68.72	16.2	
→6)-α-D-Glc*p*-(1→	5.00	3.85	3.4	3.55	3.76	3.84	4.22
B	98.28	73.52	74.00	70.54	73.00	69.28	
→3)-β-D-Glc*p*-(1→	4.64	3.24	3.74	3.39	3.46	3.72	3.90
C	103.43	74.00	85.00	69.95	76.05	61.18	
→3,6)-β-D-Glc*p*-(1→	4.53	3.53	3.72	3.41	3.83	4.07	4.22
D	102.84	73.00	85.00	68.72	75.35	69.95	
β-D-Glc*p*-(1→	4.52	3.33	3.50	3.40	3.55	3.74	3.92
E	103.08	73.52	76.25	70.54	76.05	61.18	

[a] Values of the ^{13}C chemical shifts were recorded with reference to acetone as the internal standard and fixed at δ 31.05 at 27 °C.
[b] Values of the ^{1}H chemical shifts were recorded with respect to the HOD signal fixed at δ 4.67 at 27 °C.

C-1, **D** H-3 in the HMBC experiment (Fig. 4, Table 4). The downfield shift of **A**-3 (δ 80.5) carbon signal with respect to the standard values of methyl glycosides[35,36] indicates that residue **A** is a (1→3)-linked L-fucose unit.

Residue **B** has an anomeric proton signal at δ 5.00, and the $^{3}J_{H\text{-}1,H\text{-}2}$ coupling constant is very small, but $^{1}J_{H\text{-}1,C\text{-}1}$ 170 Hz indicates that it is an α-linked moiety. Large coupling constants, $^{3}J_{H\text{-}2,H\text{-}3}$ (~10 Hz) and $^{3}J_{H\text{-}3,H\text{-}4}$ (~10 Hz) for **B** indicate that it is a D-glucosyl moiety. The downfield shifts of C-6 (δ 69.28) carbon signal with respect to the standard values indicate that residue **B** is linked at this position. These observations also support the GC–MS data for this linkage. Hence, (1→6)-linked D-glucose is present in the polysaccharide. The anomeric carbon signal of D-glucosyl moiety at δ 98.28 is further confirmed by the cross-peak **B** C-1, **C** H-3 in the HMBC experiment (Fig. 4, Table 4).

The anomeric proton signal for residue **C** is δ 4.64, and it has a large coupling constant for $^{3}J_{H\text{-}1,H\text{-}2}$ (7.9 Hz), and $^{1}J_{H\text{-}1,C\text{-}1}$ 161 Hz, indicating that it is a β-linked residue. The large $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ values (9–10 Hz) for residue **C** were observed, indicating that it is a β-D-glucosyl moiety. The downfield shift of **C**-3 (δ 85.00) with respect to the standard values indicates that residue **C** is a (1→3)-linked D-glucose moiety. The anomeric carbon signal of the D-glucose moiety was observed at δ 103.43, confirmed by the presence of cross-peak **C** C-1, **A** H-3 in the HMBC experiment (Fig. 4, Table 4).

Residue **D** has an anomeric chemical shift at δ 4.53, and $^{3}J_{H\text{-}1,H\text{-}2} \sim 7.5$ Hz indicating a β-linkage, which is further corroborated by the $^{1}J_{C\text{-}1,H\text{-}1} \sim 160$ Hz. Large coupling constants $^{3}J_{H\text{-}2,H\text{-}3}$ and $^{3}J_{H\text{-}3,H\text{-}4}$ (~10 Hz) were observed from the DQF-COSY spectrum for residue **D**, indicating that it is a D-glucosyl moiety. The anomeric carbon signal of residue **D** at δ 102.84 was confirmed by the presence of cross-peak **D** C-1, **B** H-6a, and **B** H-6b in the HMBC experiment (Fig. 4, Table 4). The downfield shift of C-3 (δ 85.00), C-6 (δ 69.95) carbon signals with respect to the standard values of methyl glycosides indicates that residue **D** is a (1→3,6)-linked D-glucose moiety.

Residue **E** (δ 4.52) was assigned to the nonreducing-end β-D-glucopyranosyl unit with coupling constants of $^{3}J_{H\text{-}1,H\text{-}2}$ (7.9 Hz), and $^{1}J_{C\text{-}1,H\text{-}1}$ (160 Hz). The large $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ values (~10 Hz) for residue **E** were observed, indicating that it is a β-D-glucosyl moiety. The carbon signals from C-1 to C-6 (Table 2) for residue **E** were identified from HMQC spectrum, and these nearly correspond to the standard values of methyl glycosides. The C-1 signal of residue **E** at δ 103.08 was confirmed by the appearance of cross-peak **E** C-1, **D** H-6a, and **D** H-6b in the HMBC experiment (Fig. 4, Table 4). Thus considering the results of the methylation analysis and the NMR experiment, it may be concluded that residue **E** is a β-glycosidically linked nonreducing-end D-glucosyl moiety.

The sequences of glycosyl residues of the polysaccharide fraction were determined from NOESY as well as ROESY experiments (Fig. 3, Table 3). Residue **A** has an NOE contact from H-1 to H-3 of residue **D**. So, residue **A** is linked at C-3 of residue **D**, indicating the following sequence:

α-L-Fuc*p*-(1→3)-β-D-Glc*p*-(1→
A **D**

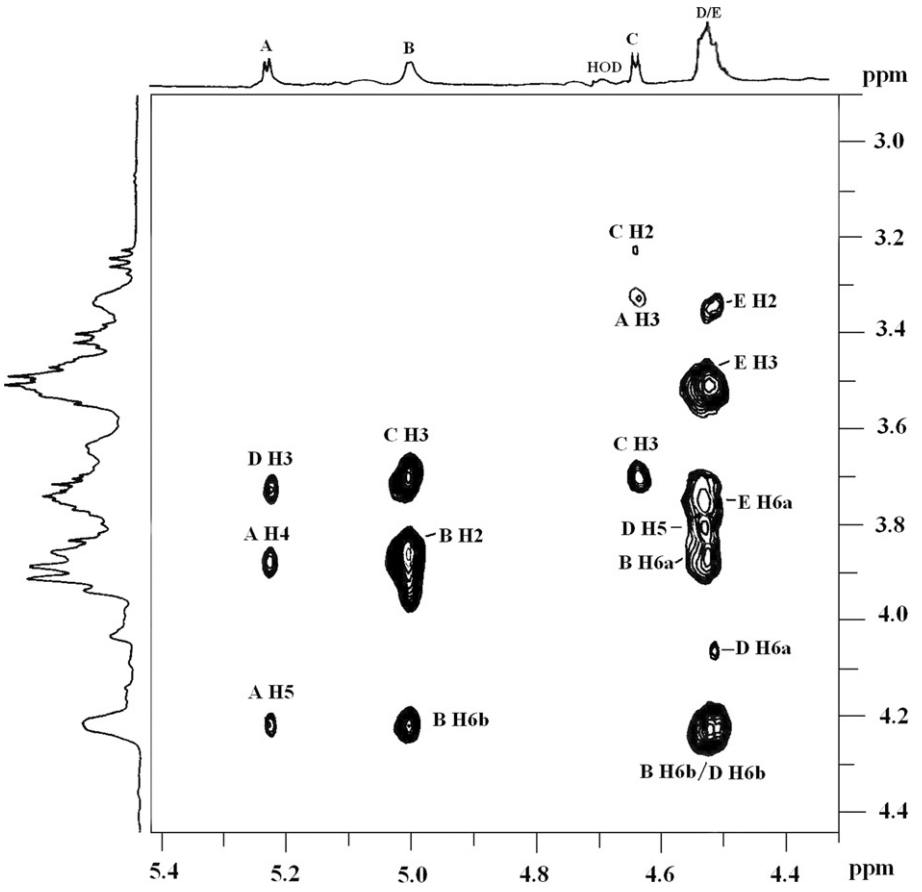


Figure 3. NOESY spectra of the polysaccharide isolated from *T. robustus*. The NOESY mixing time was 350 ms.

Table 3. NOE data for the polysaccharide isolated from *T. robustus*

Anomeric proton		NOE contact protons	
Glycosyl residue	δ	δ	Residue, atom
→3)-α-L-Fuc*p*-(1→ **A**	5.22	3.85	**A** H-4
		4.21	**A** H-5
		3.72	**D** H-3
→6)-α-D-Glc*p*-(1→ **B**	5.00	3.85	**B** H-2
		4.22	**B** H-6a
		3.74	**C** H-3
→3)-β-D-Glc*p*-(1→ **C**	4.64	3.24	**C** H-2
		3.74	**C**H-3
		3.32	**A**H-3
→3,6)-β-D-Glc*p*-(1→ **D**	4.53	3.83	**D** H-5
		3.84	**B** H-6a
		4.22	**B** H-6b
β-D-Glc*p*-(1→ **E**	4.52	3.33	**E** H-2
		3.50	**E** H-3
		4.07	**D** H-6a
		4.22	**D** H-6b

Moiety **B** has a strong interresidue contact from H-1 to H-3 of moiety **C** indicating that moiety **B** is linked to the 3-position of moiety **C**. Hence, the following sequence is assigned:

α-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→

B **C**

Residue **C** has an NOE contact from H-1 to H-3 of residue **A**, in addition to intraresidue NOE contacts to H-2 and H-3. Therefore, the following sequence is established:

→3)-β-D-Glc*p*-(1→3)-α-L-Fuc*p*-(1→

C **A**

Residue **D** has an NOE contact from H-1 to both H-6a and H-6b of residue **B**. Since H-6b of both residues **B** and **D** is present in the same signal, the connectivity between them was further confirmed by the presence of cross-coupling (**D** C-1, **B** H-6a; **D** C-1, **B** H-6b) in the HMBC experiment (Fig. 4, Table 4). Hence, moiety **D** is linked to the 6-position of moiety **B**, so the linkage in between residue **D** and **B** is as follows:

→3)-β-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→

D **B**

Residue **E** has an NOE contact from H-1 to both H-6a and H-6b of residue **D**, in addition to intraresidue NOE contacts to H-2, H-3, H-6a, indicating that residue **E** is linked to the 6-position of residue **D**, so the linkage between residues **E** and **D** is as follows:

β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→
3
↑
E **D**

Hence, from the above NOE spectrum, it is concluded that residue **A** is a 1,3-linked moiety, residue **B** a 1,6-linked, residue **C** a 1,3-linked. Furthermore, moiety **D** is 1,3,6-linked, and residue **E** is a nonreducing-end

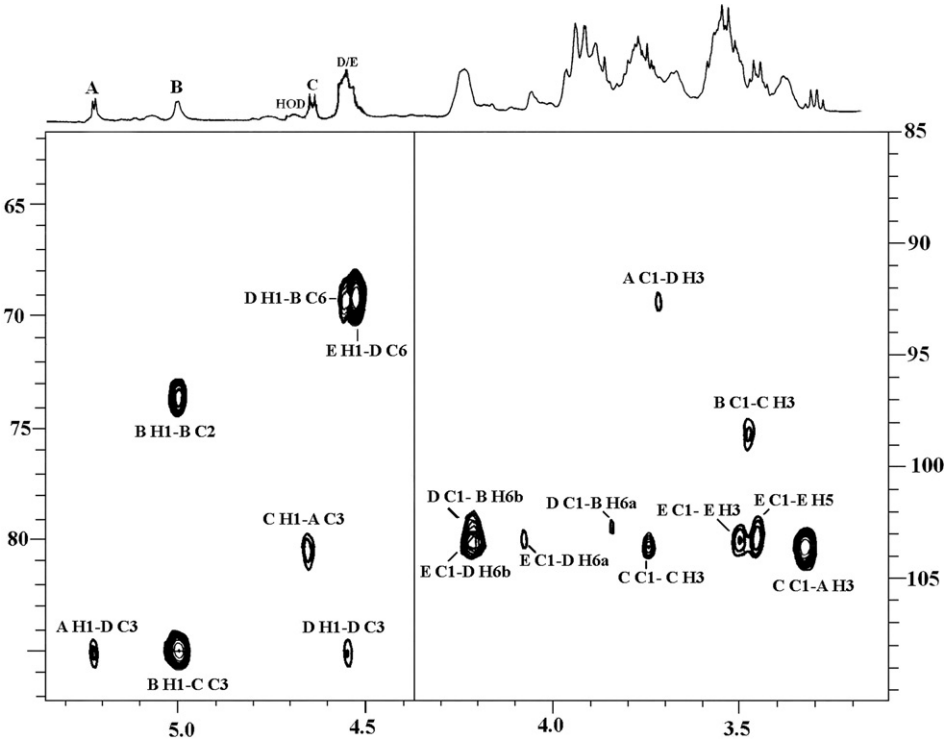
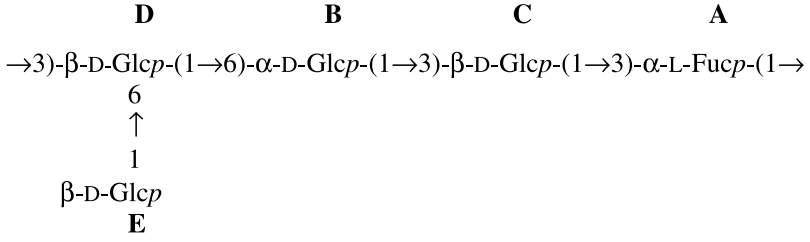


Figure 4. HMBC spectrum of the polysaccharide isolated from *T. robustus*. The delay time in the HMBC experiment was 80 ms.

Table 4. The significant $^{3}J_{H,C}$ connectivities observed in a HMBC spectrum for the anomeric protons/carbons of the sugar residues of the polysaccharide of *T. robustus*

Residue	Sugar linkage	H-1/C-1	Observed connectivities		
		δ_H/δ_C	δ_H/δ_C	Residue	Atom
A	→3)-α-L-Fuc*p*-(1→	5.22	85.00	**D**	C-3
		96.60	3.72	**D**	H-3
			1.24	**A**	H-6
B	→ 6)-α-D-Glc*p*-(1→	5.00	85.00	**C**	C-3
			73.52	**B**	C-2
		98.28	3.74	**C**	H-3
C	→3)-β-D-Glc*p*-(1→	4.64	80.50	**A**	C-3
		103.43	3.32	**A**	H-3
			3.74	**C**	H-3
D	→3,6)-β-D-Glc*p*-(1→	4.53	69.28	**B**	C-6
			85.00	**D**	C-3
		102.84	3.84	**B**	H-6a
			4.22	**B**	H-6b
E	β-D-Glc*p*-(1→	4.52	69.28	**D**	C-6
		103.08	4.07	**D**	H-6a
			4.22	**D**	H-6b
			3.50	**E**	H-3
			3.46	**E**	H-5

attached to the 6-position of residue **D**. Therefore, the following repeating unit is established:



Long-range ^{13}C–^{1}H correlation obtained from the HMBC spectrum (Fig. 4) corroborated the assigned repeating unit established from the NOESY experiments. From the HMBC experiment (Table 4), the cross-peaks of both anomeric protons and carbons of each of the sugar residues were examined, and intra- and interresidual connectivities were assigned.

Cross-peaks were found between H-1 of residue **A** (δ 5.22) and C-3 (δ 85.00) of residue **D** (**A** H-1, **D** C-3); C-1 of residue **A** (δ 92.5) and H-3 of residue **D** (**A** C-1,**D** H-3) with other intraresidual coupling between C-1 of residue **A** with its own H-6 atom. Similarly, cross-peaks between H-1 of residue **B** (δ 5.00) and C-3 (δ 85.00) of residue **C** (**B** H-1, **C** C-3); C-1 (δ 98.28) of residue **B** with H-3 (δ 3.74) of residue **C** (**B** C-1, **C** H-3) were observed. Intraresidual coupling between H-1 of **B** with its own C-2 atom was also observed. The cross-peaks between H-1 of residue **C** (δ 4.64) and C-3 (δ 80.50) of residue **A** (**C** H-1, **A** C-3); C-1 of residue **C** (δ 103.43) and H-3 (δ 3.32) of residue **A** (**C** C-1, **A** H-3) were observed with an intraresidual coupling between C-1 of residue **C** with its own H-3 atoms. The cross-peaks between H-1 of residue **D** (δ 4.53) with C-6 (δ 69.28) of residue **B** (**D** H-1, **B** C-6); C-1 of residue **D** (δ 102.84) and both H-6a and H-6b of residue **B** (**D** C-1, **B** H-6a and **B** H-6b) were observed along with other intraresidual coupling between H-1 of residue **D** with its own C-3 atom. Similarly, cross-peaks between H-1 of residue **E** (δ 4.52) and C-6 (δ 69.28) of residue **D** (**E** H-1, **D** C-6); C-1 (δ 103.08) of nonreducing sugar **E** and both H-6a (δ 3.74) and H-6b (δ 3.92) of residue **D** (**E** C-1, **D** H-6a, and **D** H-6b) were observed in addition to the intraresidual coupling between C-1 of residue **E** and its own H-3 and H-5 atoms. Thus, the appearance of these cross-peaks clearly supports the presence of the above-mentioned repeating unit in the polysaccharide, *T. robustus*.

3. Experimental

3.1. Isolation, fractionation, and purification of the crude polysaccharide

The fresh fruiting bodies of *T. robustus* (1.5 kg) were collected from the local forest, and the fruit body was

gently washed with water and then with distilled water. The mushroom bodies were pulverized for the extraction of polysaccharide by boiling with water for 6 h as applied in our earlier work.[4–6] The aqueous extract was kept overnight at 4 °C and then filtered through linen cloth. The filtrate was centrifuged at 8000 rpm at 4 °C for 30 min to obtain a clear solution, and then the polysaccharide was precipitated in 1:5 (v/v) EtOH. After keeping the precipitated material in the mixture overnight at 4 °C, it was centrifuged at 4 °C for 1 h, and then the residue was freeze-dried. The dried material was dissolved in 4% NaOH solution and reprecipitated in EtOH. The precipitated material was collected through centrifugation and dissolved in a minimum volume of water, and dialyzed through dialysis tubing of cellulose membrane (Sigma–Aldrich, retaining MW >12,400) against distilled water for 36 h to remove alkali and low-molecular-weight carbohydrate materials. The whole dialyzed solution was then centrifuged at 8000 rpm at 4 °C. The water-soluble part was freeze-dried, yielding 1.2 g of crude polysaccharide.

The crude polysaccharide was purified by gel-permeation chromatography. The polysaccharide (30 mg) was passed through Sepharose 6B column (90 × 2.1 cm) using water as the eluant with a flow rate of 0.5 mL min^{-1}. A total of 80 test tubes (2 mL each) were collected and monitored spectrophotometrically at 490 nm using the phenol–H_2SO_4 acid method.[30] A single homogeneous fraction was collected and freeze-dried, yielding 20 mg of polysaccharide. This same procedure was repeated five times to yield 100 mg of polysaccharide.

3.2. Determination of molecular weight

The molecular weight of the polysaccharide fraction was determined by a gel-chromatographic technique. Standard dextrans[29] T-200, T-70, and T-40 were passed through a Sepharose 6B column, and then the elution volumes were plotted against the logarithms of their respective molecular weights. The elution volume of the polysaccharide was then plotted in the same graph, and the molecular weight of the polysaccharide was determined.

3.3. Monosaccharide composition

The polysaccharide sample (2 mg) was hydrolyzed with 2 M trifluoroacetic acid (TFA, 1 mL) at 100 °C for 18 h in a boiling water bath. After completion of the hydrolysis, excess acid was removed by co-distillation with distilled water. The hydrolyzate was then divided into two parts. One part was examined by PC in solvent systems X and Y. Another part was converted into its respective alditol acetates by reduction with $NaBH_4$, followed by acidification with HOAc, and then co-distillation with MeOH to remove excess boric acid and drying over P_2O_5. Thereafter the whole mass was treated with pyridine and Ac_2O to prepare the desired product, which was then analyzed by GLC using columns A and B.

3.4. Paper chromatographic studies

Paper chromatographic studies were performed on Whatmann nos. 1 and 3 mm sheets. Solvent systems used were (X) BuOH–HOAc–H_2O (v/v/v, 4:1:5, upper phase) and (Y) EtOAc–pyridine–H_2O (v/v/v, 8:2:1). Alkaline silver nitrate solution[37] was used as the spray reagent.

3.5. Absolute configuration of the monosaccharides

The method used was that of Gerwig et al.[32] After the hydrolysis of the polysaccharide (1 mg) by TFA, the acid was removed by co-distillation with water. A solution of 250 μL of 0.625 M HCl in (+)-2-butanol was added to it, and the mixture was heated at 80 °C for 16 h. The reactants were then evaporated, and per-*O*-TMS-derivatives were prepared with *N*,*O*-bis(trimethylsilyl)trifluroacetamide (BSTFA). The products were analyzed by GLC using a capillary column (SPB-1, 30 m × 0.26 mm) with a temperature program (3 °C/min) from 150 ° to 210 °C. The (+)-2-butyl 2,3,4,6-tetra-*O*-TMS-glycosides obtained were identified by comparison with those prepared from the D- and L-enantiomers of the monosaccharide.

3.6. Methylation analysis

The polysaccharide (4 mg) was methylated according to the method of Ciucanu and Kerek,[33] and the product was isolated by partitioning between $CHCl_3$ and H_2O (5:1, v/v). The organic layer containing products was washed with 3 mL of water three times and dried. It was then methylated again by the Purdie method.[34] The product showed no band in the region 3600–3300 cm^{-1}. It was then hydrolyzed with 90% HCO_2H for 1 h, reduced with $NaBH_4$, acetylated with 1:1 Ac_2O–pyridine, and analyzed by GLC using columns A and B and by GLC–MS analysis using an HP-5 fused-silica capillary column.

3.7. Periodate oxidation study

The polysaccharide (10 mg) was oxidized with 0.1 M $NaIO_4$ (2 mL) at 27 °C in the dark for 48 h. The excess periodate was destroyed by adding ethylene glycol, and the solution was dialyzed against distilled water for 1 h. The product was then reduced with $NaBH_4$, neutralized with HOAc, and dried by the addition of CH_3OH. The periodate-reduced material was divided into two parts.

One part was subjected to hydrolysis, and another part was subjected to methylation following the usual procedures described above. The alditol acetates from both the hydrolyzed material and the methylated material were analyzed by GLC using columns A and B, and the latter was analyzed by GLC–MS.

3.8. Instrumental methods

Optical rotation was measured on a Perkin–Elmer model 241 MC spectropolarimeter at 25 °C. Colorimetric estimations were carried out on a Shimadzu UV–vis spectrophotometer model 1601. All gas-liquid chromatography (GLC) analyses were carried out on a Hewlett–Packard Model 5730 A gas chromatograph having a flame ionization detector. Glass columns (1.8 m × 6 mm) were packed with 3% ECNSS-M (A) on Gas Chrom Q (100–120 mesh) and 1% OV-225 (B) on Gas Chrom Q (100–120 mesh). GLC analyses were performed at 170 °C. All the GLC–MS experiments were carried out on a Hewlett–Packard 5970 MSD instrument using an HP-5 fused-silica capillary column. The program was isothermal at 150 °C; hold time 2 min, with a temperature gradient of 4 °C min^{-1} up to a final temperature of 200 °C. The IR spectrum was recorded with dried polysaccharide (1.1 mg) on a Jasco FTIR model 6200 using a solid-state ATR accessory.

The polysaccharide was dried over P_2O_5 in vacuum for several days and then deuterium exchanged five times, followed by lyophilization with D_2O (99.96%. atom ^2H, Aldrich). Then ^1H NMR and ^{13}C NMR experiments were performed with a Bruker Avance DPX-500 instrument at 27 °C. The ^1H NMR spectrum was recorded by suppressing the HOD signal (fixed at δ 4.67) using the WEFT pulse sequence.[38] The 2D-DQF-COSY experiment was carried out using standard pulse sequence at 27 °C. The TOCSY experiment was recorded at a mixing time of 60–300 ms. The NOESY and ROESY mixing delay was 350 ms. The delay time in the HMBC experiment was 80 ms. The ^{13}C NMR experiments of the polysaccharide were carried out taking acetone as the internal standard, fixing the methyl carbon signal at δ 31.05 ppm by using D_2O as the solvent.

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